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Efficient low-cost chromatin profiling with CUT&Tag

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We recently introduced Cleavage Under Targets & Tagmentation (CUT&Tag), an epigenomic profiling strategy in which antibodies are bound to chromatin proteins in situ in permeabilized nuclei. These antibodies are then used to tether the cut-and-paste transposase Tn5. Activation of the transposase simultaneously cleaves DNA and adds adapters ('tagmentation') for paired-end DNA sequencing. Here, we introduce a streamlined CUT&Tag protocol that suppresses DNA accessibility artefacts to ensure high-fidelity mapping of the antibody-targeted protein and improves the signal-to-noise ratio over current chromatin profiling methods. Streamlined CUT&Tag can be performed in a single PCR tube, from cells to amplified libraries, providing low-cost genome-wide chromatin maps. By simplifying library preparation CUT&Tag requires less than a day at the bench, from live cells to sequencing-ready barcoded libraries. As a result of low background levels, barcoded and pooled CUT&Tag libraries can be sequenced for as little as $25 per sample. This enables routine genome-wide profiling of chromatin proteins and modifications and requires no special skills or equipment.

Introduction

Development of the protocol

All dynamic processes that take place on DNA in the nucleus occur in the context of a chromatin landscape that comprises nucleosomes and their modifications, transcription factors and chromatin-associated complexes. A variety of chromatin features mark sites of transcriptional regulatory elements and regions of activation and silencing that differ between cell types and change during development and disease progression. The mapping of chromatin features genome wide has traditionally been performed using ChIP, in which chromatin is cross-linked and solubilized and an antibody to a protein or modification of interest is used to immunoprecipitate the bound DNA[1] (Fig. 1a). This basic protocol has not changed since ChIP was first described 35 years ago[2]. Rather, the enormous progress since then using ChIP is attributable to improvements in readout technologies, progressing from southern blotting to quantitative PCR, to microarrays, and over the past decade to high-throughput sequencing (ChIP-seq). ChIP-seq remains challenging with small samples and is fraught with signal-to-noise ratio issues and artefacts. However, recent modifications of the basic strategy have greatly increased resolution[3–6] and efficiency[7–9].

An alternative chromatin profiling strategy that is becoming increasingly popular is enzyme tethering in situ, whereby the chromatin protein or modification of interest is targeted by an antibody or fusion protein, and the underlying DNA is marked or released. A succession of enzyme-tethering methods have been introduced over the past two decades, including DamID[10], ChEC (Chromatin Endogenous Cleavage)[11] and ChIC (Chromatin ImmunoCleavage)[11]. In DamID, expression of a fusion between a chromatin protein of interest and *Escherichia coli* Dam methyltransferase results in targeted DNA methylation of GATC motifs near sites of binding, and a GATC-specific restriction enzyme is used to cleave fragments for mapping. In ChEC and ChIC, micrococcal nuclease (MNase) is tethered to a target protein either directly as a fusion protein (ChEC) or indirectly to an antibody via a protein A–MNase fusion protein (ChIC). Addition of calcium ions activates MNase to cleave and release the targeted DNA fragments for DNA sequencing. Both strategies have been adapted for a sequencing readout (ChEC-seq and CUT&RUN/ChIC-seq)[12,13] (Fig. 1b). CUT&RUN has been fully automated for high-throughput application[14]. The improved signal-to-noise ratio of CUT&RUN relative to ChIP-seq translates to an order-of-magnitude reduction in the amount of sequencing

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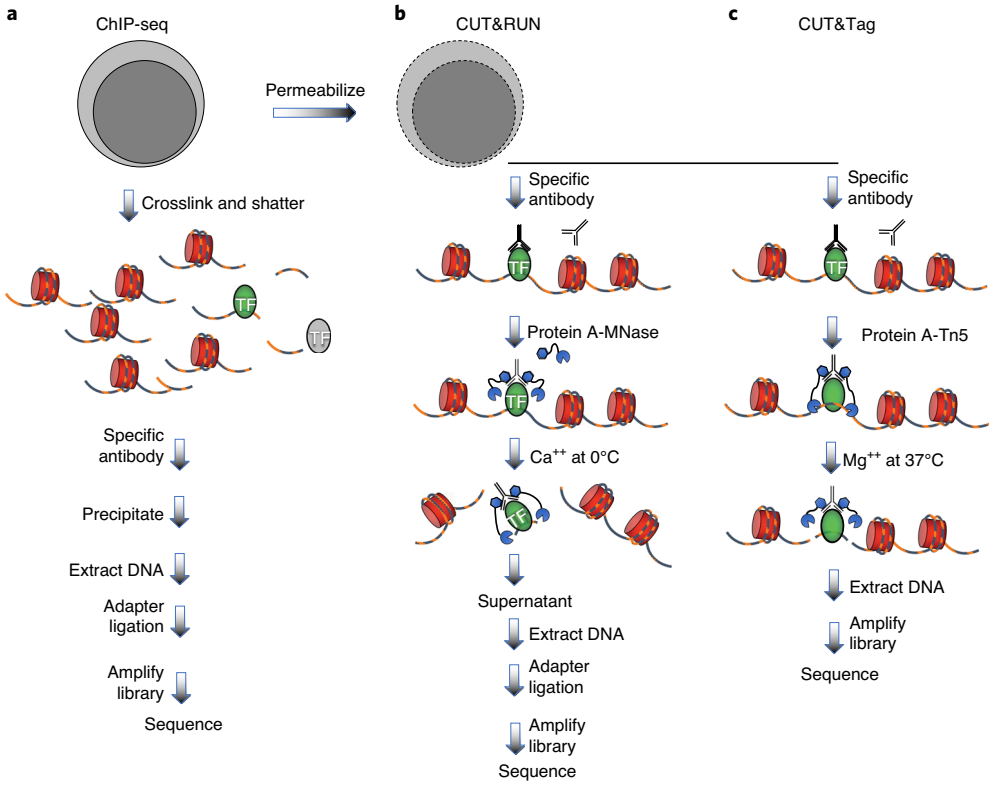


Fig. 1 | Steps in antibody-targeted chromatin profiling strategies. a, ChIP-seq, **b**, CUT&RUN, **c**, CUT&Tag. Cells are indicated in gray, chromatin as red nucleosomes, and a specific chromatin protein in green. See text for details of each procedure.

required to map chromatin features. Unlike ChIP, which requires cross-linking, CUT&RUN is performed on intact unfixed cells or nuclei, and so is free of epitope masking and other artefacts attributable to the harsh conditions required for ChIP. Importantly, the high efficiency of CUT&RUN makes it suitable for much lower cell numbers than is practical with ChIP-seq[15]. These and other advantages of CUT&RUN have resulted in a surge in popularity of the method since its introduction in 2017, replacing ChIP-seq for many chromatin profiling applications[16–19], including for single-cell analysis[17]. During this time, novel computational tools have been introduced to take advantage of the near base-pair resolution and low background levels of CUT&RUN, including CUT&RUNTools[20], Sparse Enrichment Analysis for CUT&RUN (SEACR)[21] and EChO[22].

A very recent development in enzyme-tethering chromatin profiling technologies has been the substitution of the Tn5 transposase for MNase in CUT&RUN, which we refer to as CUT&Tag[23] (Fig. 1c). The protein A-Tn5 (pA-Tn5) transposome, when loaded with mosaic end adapters and activated by magnesium ions, integrates the adapters into nearby DNA to create fragments that are amplifiable to generate sequencing libraries. Antibody-tethered Tn5-based methods, not only CUT&Tag[23], but also ChIL-seq[24], ACT-seq[25] and CoBATCH[26], achieve high sensitivity owing to the high efficiency of tethered Tn5 integration. Although these methods are based on the same principle and all have been used for single-cell profiling, there are important differences in the protocols that can result in different outcomes. Like CUT&Tag, ACT-seq and CoBATCH use a pA-Tn5 fusion protein, whereas ChIL-seq uses a secondary antibody conjugated to a double-stranded template for Tn5 transposome binding and linear amplification by T7 RNA polymerase. As Tn5 remains bound following the cut-and-paste tagmentation reaction, fragments are retained within the cell, making these methods suitable for single-cell profiling. Indeed, these methods were introduced with proof-of-concept single-cell profiling data, suggesting that this basic strategy represents an important future direction for single-cell chromatin profiling in studies of development and disease.

CUT&Tag builds on the CUT&RUN protocol, in which cells are permeabilized and incubated with a primary antibody. Then a secondary antibody and pA-Tn5 are successively tethered to antibody-bound sites. Stringent washing with 300 mM NaCl is critical to limit the affinity of Tn5 for exposed

DNA. Here we describe the need for controlling background Tn5 affinity for accessible DNA and describe how our CUT&Tag protocol effectively suppresses this artefact for unambiguous mapping of chromatin epitopes. We present a protocol that can process either native or fixed nuclei, and includes alternative methods for DNA isolation. To illustrate the method, we describe a typical experiment, including evaluation of the results. Further, we validate a single-tube format for CUT&Tag that requires no DNA isolation but instead uses tagmented material directly for library amplification. We document critical steps for the CUT&Tag protocol informed by our experiences helping users establish this method in their research.

Applications of the method

In this protocol we describe CUT&Tag for bench-top application, suitable for profiling a dozen or so samples in a day. The major practical advantage of CUT&Tag over other methods is that it eliminates the time and expense of preparing sequencing libraries, but CUT&Tag also has other important attributes that makes it the protocol of choice for most chromatin profiling applications. CUT&Tag has an improved signal-to-noise ratio for histone marks, at least in part because an antibody-tethered Tn5 integrates its mosaic-end adapters and remains bound during the incubation. We also find that CUT&Tag is more efficient than traditional chromatin profiling methods. This is most likely because integration by targeted Tn5 is more efficient than enzymatic end-polishing and ligation in traditional library preparation steps. The higher efficiency of tagmentation and the retention of targeted Tn5-bound particles makes CUT&Tag preferable for single-cell profiling. We have previously described this application using the Takara ICELL8 robotic nanowell chip system[23].

Although we are excited by the prospects for routine single-cell CUT&Tag (scCUT&Tag) chromatin profiling, all current single-cell profiling methods produce sparse data, and this translates into orders-of-magnitude lower information content per dollar spent than the simple bench-top protocol described here. CUT&Tag profiling identifies of the order of 10,000 high-quality chromatin features, such as promoter marks, for as little as $25 per sample, which places the method in the realm of clinical sample testing. We previously described a fully automated CUT&RUN protocol[14], which is currently in operation as a core facility at the Fred Hutchinson Cancer Research Center serving researchers and clinicians. Adaptation of this CUT&Tag protocol for automation is an attractive prospect.

Comparison with other methods

Compared to ChIP-seq, both CUT&RUN[15] and CUT&Tag[23] require less input material and fewer reads to map features. These methods have a higher signal-to-noise ratio, have little or no fragmentation bias, and are amenable to calibration to equalize samples in a series for comparison. By eliminating library preparation, CUT&Tag, like ATAC-seq, requires less time and effort than the other methods to produce libraries that are ready for sequencing. Although ATAC-seq can be performed in just a few hours, CUT&Tag requires additional incubation and wash steps that require a day. However, because fewer reads are required for peak detection, sequencing of pooled CUT&Tag active chromatin libraries is less expensive than performing ChIP-seq or ATAC-seq on the same samples. This becomes especially critical in low-cell-number applications.

Experimental design

Overview

The CUT&Tag method for in situ tagmentation of chromatin complexes can be performed on the bench-top and completed in a day using standard laboratory equipment. Our detailed protocol applies to any chromatin feature for which an antibody is available and should be adaptable to any cell line, primary cell or tissue for which there is a standard isolation protocol. In brief, native or cross-linked nuclei are prepared and immobilized on magnetic beads (Steps 1–16). Beads are incubated with a primary antibody, followed by incubation with a secondary antibody to increase the number of IgG molecules at each epitope bound by the primary antibody (Steps 17–28). Beads are washed and incubated with pA-Tn5 loaded with mosaic-end adapters (Steps 29–34) and washed under stringent conditions. Tn5 is activated by addition of Mg^{2+}, whereupon integration of adapters effectively inactivates the pA-Tn5 transposome, so that tagmentation will reach completion during the incubation period. Therefore, timing is not as critical for pA-Tn5 as for pA-MNase used in CUT&RUN, in which the nuclease continues to cleave DNA in the presence of Ca^{2+}. A small amount

of residual *E. coli* DNA that is carried over from pA-Tn5 purification becomes tagmented and serves as a calibration standard in lieu of a spike-in for comparing samples in an experimental series[23,27]. DNA is extracted with phenol-chloroform (Step 35A) or released in a small volume of SDS and then mixed with Triton-X100 to neutralize the SDS (Step 35B). Samples are enriched by PCR amplification (Steps 36 and 37) and a single solid-phase reversible immobilization (SPRI) magnetic bead cleanup step (Steps 38–45). Up to 96 barcoded libraries from multiple experiments may be pooled for a two-lane flow cell, as 3 million mapped paired-end reads are usually sufficient for a genome-wide profile of a histone modification in human cells.

Controls

For CUT&Tag we recommend using a positive control antibody that targets an abundant epitope. Therefore the library DNA can be easily detected following amplification. For histone modifications, a nucleosomal ladder is expected by TapeStation or other capillary electrophoretic analysis method. Once the expected library DNA pattern is observed by capillary electrophoresis for a positive control such as the H3K27me3 histone modification, it is not necessary to sequence this sample. As a negative control, we recommend simply omitting the primary antibody so that the secondary antibody will randomly coat the chromatin at low efficiency without sequence bias. Although the negative control is useful for ascertaining the success of an experiment by capillary gel analysis, sequencing it is optional because, for large genomes, the reads are too sparsely distributed to be useful for data analysis. An exception is peak-calling, such as using SEACR (https://seacr.fredhutch.org), where comparing each experimental sample versus a negative control is the preferred option.

Limitations

As is the case with ChIP-seq and CUT&RUN, CUT&Tag relies on the affinity of the primary antibody for its target and its specificity under the conditions used for binding. Although low background levels reduce read-depth requirements and thus lower costs, antibody-specific problems such as low affinity and epitope masking will require deeper sequencing to identify features. Unlike ChIP-seq, in which antibodies bind their epitopes in solution, CUT&RUN and CUT&Tag bind chromatin targets in situ. Therefore, we expect that antibodies successfully tested for specificity by immunofluorescence (IF) are likely to work.

Whereas CUT&RUN provides base-pair resolution that can be especially valuable for mapping transcription factors[22], the pA-Tn5 complex is bulkier than MNase[28] and steric effects evidently reduce resolution. Furthermore, the requirement for more stringent washes to avoid binding to and tagmentation of accessible DNA also reduces occupancy of transcription factors (TFs) in unfixed cells, making CUT&RUN preferable for both resolution and signal-to-noise ratio for profiling many TFs. In addition, CUT&RUN has been used to distinguish direct DNA binding from nucleosome binding in characterizing pioneer transcription factors[22], and for detecting 3D contact sites[13], but these applications have not been demonstrated for CUT&Tag.

As CUT&Tag is a new method, there are no bioinformatics tools expressly designed for it. However, widely used data processing tools including bowtie2 (ref. [29]) for alignment, bedtools[30] and Picard tools (https://github.com/broadinstitute/picard) for analysis, and Deeptools[31] for heatmaps, are recommended for these tasks. Also, we expect that tools designed for CUT&RUN, including SEACR[21] and CUT&RUNTools[20] will work for CUT&Tag.

Materials

Biological materials

- Cell suspension. We have used human K562 (RRID: CVCL_0004) and other mammalian cell lines, Drosophila S2 cells (RRID: CVCL_IZ08) and dissected *Drosophila* tissues such as brains and imaginal discs **! CAUTION** The cell lines used in your research should be regularly checked to ensure they are authentic and are not infected with mycoplasma.

Reagents

- Distilled, deionized or RNAse-free H_2O (dH_2O; Promega, cat. no. P1197)
- 1 M HEPES (K+) pH 7.9 (Sigma-Aldrich, cat. no. H3375)
- 1 M Potassium chloride (KCl; Sigma-Aldrich, cat. no. P3911)
- 10% (vol/vol) Triton-X100 (Sigma-Aldrich, cat. no. X100)
- 2 M Spermidine (Sigma-Aldrich, cat. no. S2501)

- Glycerol (Sigma-Aldrich, cat. no. G5516)
- Roche Complete Protease Inhibitor EDTA-Free tablets (Sigma-Aldrich, cat. no. 5056489001)
- 1 M HEPES (Na+) pH 7.5 (Sigma-Aldrich, cat. no. H3375)
- 0.5 M EDTA (Research Organics, cat. no. 3002E)
- 5 M Sodium chloride (NaCl; Sigma-Aldrich, cat. no. S5150-1L)
- 30% (wt/vol) BSA (Sigma-Aldrich, cat. no. A8577)
- Dimethyl sulfoxide (DMSO; Sigma-Aldrich cat. no. D4540)
- Concanavalin-coated magnetic beads (Bangs Laboratories, cat. no. BP531)
- 1 M Manganese chloride ($MnCl_2$; Sigma-Aldrich, cat. no. 203734)
- 1 M Calcium chloride ($CaCl_2$; Fisher, cat. no. BP510)
- Antibody to a chromatin epitope of interest, e.g., α-H3K4me3 rabbit polyclonal antibody (Active Motif, cat. no. 39159, RRID: AB_2616029)
- Positive control antibody to an abundant epitope, e.g., anti-H3K27me3 rabbit monoclonal antibody (Cell Signaling Technology, cat. no. 9733, RRID: AB_2615077)
- Secondary antibody to increase the number of IgG molecules per targeted epitope, e.g., guinea pig anti-rabbit (Antibodies-Online, cat. no. ABIN101961, RRID: AB_10775589) or rabbit anti-mouse (Abcam, cat. no. ab46540, RRID: AB_2614925 ▲**CRITICAL** The immunoglobin-binding moiety of pA-Tn5 binds tightly only to certain IgG isotypes of some host species. Thus, the secondary amplifying antibody must be chosen to provide high affinity for protein A.
- pA-Tn5 fusion protein (loaded enzyme stored in 50% (vol/vol) glycerol). See https://www.protocols.io/view/3xflag-patn5-protein-purification-and-meds-loading-8yrhxv6. The 3 × Flag-pA-Tn5-Fl (pA-Tn5) plasmid is available from Addgene (cat. no. 124601). Alternatively, pAG-Tn5 (Protein A/G fused to Tn5) is commercially available for use with this protocol (e.g., https://www.epicypher.com/products/featured-products/cutana-pag-tn5-for-chic-cut-tag-50-rxns). ▲**CRITICAL** May be stored at −20 °C for at least a year. pA-Tn5 is loaded with oligonucleotide adapters with 19mer Tn5 mosaic ends. Adapters are generated by annealing Mosaic end_Adapter A (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG) and Mosaic end_Adapter B (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG) with Mosaic end_reverse ([PHO]CTGTCTCTTATACACATCT)[32].
- 100 mM Magnesium chloride ($MgCl_2$; Sigma-Aldrich, cat. no. M8266-100G)
- 10% SDS (Sigma-Aldrich, cat. no. L4509)
- NEBNext High-Fidelity 2 × PCR Master Mix (cat. no. M0541L)
- Proteinase K (Thermo Fisher Scientific, cat. no. EO0492; for option A)
- Phenol-chloroform-isoamyl alcohol 25:24:1 (PCI; Invitrogen, cat. no. 15593049; for option A) **!CAUTION** Phenol and chloroform are toxic, so they should be handled in a hood while wearing disposable gloves.
- Chloroform (Sigma, cat. no. 366919-1L; for option A)
- 1 M TAPS (to pH 8.5 with NaOH; for option B)
- 0.33% (vol/vol) Triton-X100 (Sigma-Aldrich, cat. no. X100; for option B)
- SPRI magnetic beads (Agencourt AMPure XP, Beckman Coulter, cat. no. A63880)
- 1 M Tris-HCl pH 8.0 (Fisher, cat. no. BP1521)
- Ethanol (Decon Labs, cat. no. 2716)
- PCR primers. 10 μM stock solutions of a universal i5 primer and 16 i7 primers with unique barcodes by Buenrostro et al.[33]. Sequences are listed in Supplementary Table 1. We order oligonucleotides from IDT in salt-free format, Oligonucleotides are dissolved in water and stored as 100 mM and 10 mM stock solutions at 4 °C for at least 2 years; higher purification formats are optional. Our PCR conditions are optimized for these custom primers **!CAUTION** Do not use Nextera primers, which will not work efficiently.
- Qubit dsDNA HS kit (Life Technologies, cat. no. Q32851)

Equipment

- Centrifuge, swing bucket (Eppendorf, model no. 5810)
- Centrifuge, fixed angle rotor (Eppendorf, model no. 5424)
- Centrifuge, refrigerated fixed angle rotor (Eppendorf, model no. 5415R)
- Macsimag magnetic separator (Miltenyi, cat. no. 130-092-168), which allows clean withdrawal of the liquid from the bottom of 0.5, 1.5 and 2 mL microfuge tubes
- Vortex mixer (VWR Vortex Genie)

- Microcentrifuge (VWR Model V)
- 15 mL or 50 mL Conical centrifuge tubes
- Cryogenic screw-cap vials (Corning, cat. no. 430658)
- Mr. Frosty containers (Thermo, cat. no. 5100-0001)
- 0.5 mL Thin-wall PCR tubes (Axygen, Fisher, cat. no. 14-222-292)
- 1.5-mL Microcentrifuge tubes (Genesee, cat. no. 22-282)
- 2-mL Microcentrifuge tubes (Axygen, cat. no. MCT-200-C)
- Tube rotator (Labquake, Thermo Fisher)
- Heater block with wells for 1.5-mL microcentrifuge tubes
- Water baths (set to 37 °C and 70 °C)
- MaXtract phase-lock microcentrifuge tubes (Qiagen, cat. no. 139046)
- Capillary electrophoresis instrument (Agilent Tapestation 4200)
- Qubit Fluorometer (Life Technologies, cat. no. Q33216)

Software

- Bowtie2 version 2.2.5 (http://bowtie-bio.sourceforge.net/bowtie2/index.shtml)
- https://github.com/Henikoff/Cut-and-Run
- Picard version 2.15 (https://broadinstitute.github.io/picard/index.html)
- SEACR peak-caller is available as a public web server (https://seacr.fredhutch.org) or as scripts from https://github.com/FredHutch/SEACR/

Reagent setup

NE1 buffer

Mix 1 mL 1M HEPES-KOH pH 7.9, 500 µL 1M KCl, 12.5 µL 2 M spermidine, 500 µL 10% (vol/vol) Triton-X100, and 10 mL glycerol in 38 mL dH_2O, and add 1 Roche Complete Protease Inhibitor EDTA-Free tablet. Chill on ice before use. Store the buffer at 4 °C for 3 months.

Wash buffer

Mix 1 mL 1 M HEPES pH 7.5, 1.5 mL 5 M NaCl, 12.5 µL 2 M spermidine, bring the final volume to 50 mL with dH_2O, and add 1 Roche Complete Protease Inhibitor EDTA-Free tablet. Store the buffer at 4 °C for up to 1 month.

Binding buffer

Mix 200 µL 1M HEPES-KOH pH 7.5, 100 µL 1M KCl, 10 µL 1M $CaCl_2$ and 10 µL 1M $MnCl_2$, and bring the final volume to 10 mL with dH_2O. Store the buffer at 4 °C for 6 months.

Antibody buffer

Mix 8 µL 0.5 M EDTA and 6.7 µL 30% BSA with 2 mL Wash buffer and chill on ice. Make fresh.

300-Wash buffer

Mix 1 mL 1 M HEPES pH 7.5, 3 mL 5 M NaCl and 12.5 µL 2 M spermidine, bring the final volume to 50 mL with dH_2O and add 1 Roche Complete Protease Inhibitor EDTA-Free tablet. Store at 4 °C for up to a month.

Tagmentation buffer

Mix 1 mL 300-wash buffer and 10 µL 1 M $MgCl_2$ (to 10 mM). Make fresh.

Post-tagmentation wash buffer

Mix 10 µL 1 M TAPS pH 8.5 in 1 mL dH_2O (to 10 mM). Make fresh.

SDS release buffer

Mix 10 µL 10% SDS and 10 µL 1 M TAPS pH 8.5 in 1 mL dH_2O. Make fresh.

Tn5-adapter complex formation

1 Anneal each of Mosaic end adapter A (ME-A) and Mosaic end adapter B (ME-B) oligonucleotides with Mosaic end–reverse oligonucleotides[25].

2 To anneal, dilute oligonucleotides to 200 μM in annealing buffer (10mM Tris pH8, 50mM NaCl, 1 mM EDTA). Each pair of oligos, ME-A+ME-Reverse and ME-B+ME-Reverse, is mixed separately resulting in 100 μM of annealed product.
3 Place the tubes in a 90–95 °C hot block and leave for 3–5 min, then remove the hot block from the heat source, allowing slow cooling to room temperature (~45 min).
4 Mix 16 μL of 100 μM equimolar mixtures of preannealed ME-A and ME-B oligonucleotides with 100 μL of 5.5 μM pA-Tn5 fusion protein.
5 Incubate the mixture on a rotating platform for 1 h at room temperature and then store at −20 °C for up to 1 year. ▲**CRITICAL STEP** pA-Tn5 aliquots received from the CUT&RUN team are pre-loaded with adapters suitable for single- or dual-indexing on a paired-end Illumina flow-cell platform.

Procedure

▲**CRITICAL** The following procedure is for 16 samples and can be scaled up or scaled down by altering volumes at all steps proportionally.
▲**CRITICAL** Step 35A requires tube transfers for DNA purification prior to PCR, including handling toxic materials. Option B is performed in single PCR tubes and requires no toxic materials. We have obtained high quality data for low cell numbers using either option.

Prepare concanavalin A beads ● Timing 15 min

1 Resuspend and withdraw enough of the ConA bead slurry such that there will be 10 μL (option A) or 3 μL (option B) for each final sample of up to ~500,000 mammalian cells (option A) or up to ~100,000 cells (option B).
2 Transfer 160 μL (option A) or 55 μL (option B) ConA bead slurry into 1.5 mL binding buffer in a 2 mL tube, mix by pipetting, and place the tube on a magnet stand to allow the mixture to clear (30 s–2 min).
3 Withdraw the liquid completely, and remove from the magnet stand. Add 2 mL binding buffer and mix by pipetting.
4 Place the tube on a magnet stand to allow the mixture to clear, withdraw liquid, resuspend in 160 μL (option A) or 55 μL (option B) binding buffer, and hold on ice until nuclei are ready (freshly cross-linked or thawed).

Prepare nuclei, optionally fix and cryopreserve ● Timing 1 h

5 Transfer a fresh culture of up to 20 million cells to a conical centrifuge tube (15 mL or 50 mL) at room temperature and count cells. This protocol can be used for up to ~500,000 mammalian (e.g., human K562) cells to be sequenced per sample.
▲**CRITICAL STEP** The single-tube protocol (option B) should only be used with nuclei, with an upper limit of ~100,000 starting cells per sample.
▲**CRITICAL STEP** Cross-linking is not required for CUT&Tag and can cause epitope masking. However, for this protocol light cross-linking to fix nuclei may be beneficial, because it helps keep nuclei intact and reduces clumping throughout the procedure.
6 Centrifuge for 3 min at 600*g* in a swing-bucket rotor at room temperature and drain liquid.
7 Resuspend in 1 volume PBS at room temperature while pipetting.
8 Centrifuge for 3 min at 600*g* in a swing-bucket rotor at room temperature and drain liquid.
9 Resuspend in ½ volume (relative to the starting culture) ice-cold NE1 with gentle vortexing. Allow to sit on ice for 10 min.
10 Centrifuge for 4 min at 1,300*g* at 4 °C in a swing-bucket rotor and drain liquid by pouring off, then inverting onto a paper towel for a few seconds.
11 Resuspend in ½ volume of PBS (relative to starting culture). For unfixed nuclei, skip to Step 14.
12 While gently vortexing add 16% formaldehyde to 0.1% (e.g., 62 μL–10 mL) and incubate at room temperature for 2 min.
13 Stop cross-linking by addition of 2.5 M glycine to twice the molar concentration of formaldehyde (e.g., 300 μL–10 mL).
14 Centrifuge for 4 min at 1,300*g* at 4 °C and drain the liquid by pouring off, then inverting onto a paper towel for a few seconds.

15 Resuspend in wash buffer to a concentration of ~1 million cells per mL. Count nuclei using a ViCell, cell counter slide or equivalent.

16 (Optional) Nuclei may be slow-frozen by aliquoting 900 µL into cryogenic vials containing 100 µL DMSO, mixed, then placed in a Mr. Frosty container filled to the line with isopropanol and placed in a −80 °C freezer overnight for storage at −80 °C indefinitely.

Bind nuclei to ConA beads ● Timing 15 min

17 Use fresh nuclei on ice or thaw the frozen nuclei aliquot at room temperature, for example by placing in a 20 mL beaker of water.

18 Mix 50–200 µL of cell suspension with 10 µL (option A) or 3 µL (option B) beads (from Step 4) in thin-walled (0.5 mL) PCR tubes and allow to sit at room temperature for 10 min.

19 Place the tubes on a magnet stand to allow the mixtures to clear and withdraw and discard the liquid.
▲ **CRITICAL STEP** Surface tension will cause bead-bound cells to slide down to the bottom of the tube, so to avoid losses here and below, set the pipettor at 45 µL for a 50 µL volume.
▲ **CRITICAL STEP** If loss of bead-bound cells is occurring during liquid withdrawal for single-tube CUT&Tag (option B), then up to 5 µL of ConA beads may be used without causing PCR inhibition.
▲ **CRITICAL STEP** If desired, the supernatants may be counted on a ViCell, cell counter slide or equivalent to check efficiency of nuclei binding to beads, which is usually >90%.

Bind primary antibody ● Timing 1 h 15 min

▲ **CRITICAL** To evaluate the success of the procedure by capillary electrophoresis (e.g., TapeStation) analysis, include in parallel a positive control antibody (e.g., anti-H3K27me3) and a no-primary antibody negative control.

20 Resuspend cells in 50 µL antibody buffer pre-mixed with antibody (1:100) with gentle vortexing.
▲ **CRITICAL STEP** For bulk processing of up to 16 samples, resuspend in antibody buffer and antibody (1:100) with gentle vortexing. We use 1:100 by default or the manufacturer's recommended concentration for IF.
? TROUBLESHOOTING

21 Place on a rotator at room temperature and incubate for at least 1 h or at 4 °C overnight.
■ **PAUSE POINT** Antibody incubation may proceed on a rotator overnight at 4 °C. We have not noticed any difference between the efficiency of a 1–2 h room temperature incubation and an overnight 4 °C incubation.

Bind secondary antibody ● Timing 45 min

▲ **CRITICAL** The secondary antibody step is required for CUT&Tag to increase the number of protein A binding sites for each chromatin target. We have found that without the secondary antibody the efficiency of tagmentation is very low.

22 After a quick spin (~100*g*, 1 s, room temperature), place each tube on the magnet stand to allow the mixtures to clear and withdraw and discard the liquid.
▲ **CRITICAL STEP** A quick spin on a microcentrifuge (~100*g*, 1 s, room temperature) will minimize carryover of reagents.

23 Mix secondary antibody 1:100 in wash buffer and squirt in 50 µL per sample while gently vortexing to allow the solution to dislodge the beads from the sides.

24 Place the tubes on a rotator at room temperature for 30 min.
■ **PAUSE POINT** Secondary antibody incubation may proceed overnight at 4 °C.

25 After a quick spin (~100*g*, 1 s, room temperature), place the tubes on a magnet stand to allow the mixtures to clear and withdraw and discard the liquid.

26 With tubes still on the magnet stand, carefully add 500 µL wash buffer. The surface tension will cause the beads to slide up along the side of the tube closest to the magnet.

27 Slowly withdraw the liquid with a 1 mL pipette tip and discard.
▲ **CRITICAL STEP** To withdraw the wash liquid without losing beads, set the pipettor to 600 µL, and keep the plunger depressed while lowering the tip to the bottom. The liquid level will rise to near the top completing the wash. Then ease off on the plunger until all the liquid is withdrawn, and remove the pipettor. When using low-retention PCR tubes, a strong magnet stand such as the Macsimag is recommended to minimize loss of beads during this and other washing steps.

28 After a quick spin (~100*g*, 1 s, room temperature), place the tubes on a magnet stand to remove the last drop with a 20 µL pipettor.

Bind pA-Tn5 adapter complex ● Timing 1 h 15 min

29 Mix pA-Tn5 adapter complex in 300-wash buffer to a final concentration of 1:200 (or follow the manufacturer's recommendation).

30 Squirt in 50 µL per sample of the pA-Tn5 mix while gently vortexing to allow the solution to dislodge most or all of the beads.

31 Place the tubes on a rotator at room temperature for 1 h.

32 After a quick spin (~100*g*, 1 s, room temperature), place the tubes on a magnet stand to allow the mixtures to clear and pull off the liquid, setting the pipettor to 45 µL to avoid losses.

33 With the tubes still on the magnet stand, carefully add 500 µL 300-wash buffer.

34 Remove the liquid, and after a quick spin (~100*g*, 1 s, room temperature), place the tubes on a magnet stand to remove the last drop with a 20 µL pipettor, and proceed immediately to the next step.

Tagmentation and DNA purification

35 To tagment and extract the DNA, follow option A. For single-tube CUT&Tag, follow option B. Both options provide similar results.

(A) **Tagmentation with DNA extraction ● Timing 3 h**

(i) Add 200 µL 300-wash buffer. Invert 10× to allow the solution to dislodge most or all of the beads.

(ii) After a quick spin (~100*g*, 1 s, room temperature), place the tubes on a magnet stand to allow the mixtures to clear and pull off the liquid.

(iii) Add 300 µL tagmentation buffer while gently vortexing.

(iv) Incubate at 37 °C for 1 h.

▲ **CRITICAL STEP** It is typical for the beads to form a large clump during incubation owing to the viscoelasticity of DNA. However, for abundant chromatin epitopes, extensive fragmentation of the genome will normally result in reduced clumping and release of beads into suspension, turning the liquid brownish relative to samples profiling less abundant epitopes and to negative controls.

(v) Add 10 µL 0.5M EDTA, 3 µL 10% SDS and 2.5 µL 20 mg/mL Proteinase K to each sample. Mix by inversion and incubate for 10 min at 70 °C.

(vi) Add 300 µL PCI and mix by full-speed vortexing ~2 s.

! **CAUTION** Phenol and chloroform are toxic, so they should be handled wearing disposable gloves in a hood.

(vii) Transfer the solution to a phase-lock tube, and centrifuge for 5 min at room temperature at 16,000*g*.

(viii) Add 300 µL chloroform, invert ~10× to mix, and centrifuge for 5 min at room temperature at 16,000*g*.

(ix) Remove the aqueous phase by pipetting to a fresh 1.5 mL Eppendorf tube containing 1 mL 100% ethanol and mix by vortexing or tube inversion.

(x) Chill on ice for 5 min and centrifuge for at least 10 min at 4 °C at 16,000*g*.

(xi) Pour off the liquid and drain on a paper towel.

(xii) Rinse the pellet by adding 1 mL 80–100% ethanol, invert ~10× to mix, and centrifuge for 1 min at 4 °C at 16,000*g*.

(xiii) Carefully pour off the liquid and drain on a paper towel. Air dry until the ethanol evaporates (~5 min for 100%).

(xiv) Dissolve the pellet in 21 µL 1 mM Tris-HCl at pH8 and 0.1 mM EDTA.

(xv) Vortex hard and after a quick spin transfer the solution to a 0.5 mL thin-walled PCR tube.

▲ **CRITICAL STEP** Do not add glycogen, which will inhibit the PCR.

▲ **CRITICAL STEP** Do not perform TapeStation analysis at this stage as the tagmented DNA is not yet released into solution.

(B) **Single-tube tagmentation and pA-Tn5 release ● Timing 2 h 30 min**

(i) Add 50 µL 300-wash buffer to resuspend the bead/nuclei pellet by inversions or vortexing.

(ii) After a quick spin (~100*g*, 1 s, room temperature), place the tubes on a magnet stand to allow the mixtures to clear and pull off the liquid, setting the pipettor to 45 µL to avoid losses.

(iii) After a second quick spin (~100*g*, 1 s, room temperature), place the tubes on a magnet stand to remove the last drop with a 20 µL pipette tip.

(iv) Resuspend the bead/nuclei pellet in 50 µL tagmentation buffer with gentle vortexing.

(v) Incubate at 37 °C for 1 h in a PCR cycler with heated lid.
(vi) Place the tubes on a magnet stand and remove the liquid.
(vii) After a quick spin (~100*g*, 1 s, room temperature), return the tubes to the magnet stand and remove any remaining liquid using a 20-µL pipette tip.
(viii) Resuspend the beads in 50 µL 10 mM TAPS buffer and mix.
(ix) After a quick spin (~100*g*, 1 s, room temperature), place the tubes on a magnet stand and remove the liquid.
(x) After a second quick spin (~100*g*, 1 s, room temperature), return the tubes to the magnet stand and remove any remaining liquid using a 20 µL pipette tip.
(xi) Resuspend the beads in 5 µL (0.1%) SDS release buffer.
▲ **CRITICAL STEP** Use a 20 µL pipette tip to dispense while wetting the sides of the tubes to recover the fraction of beads sticking to the sides (which can be large for unfixed nuclei). During and after dispensing the liquid 'twirl' the tube between thumb and forefinger with the tip rubbing along the side. Do not withdraw liquid with the pipettor.
(xii) After a quick spin, incubate at 58 °C for 1 h in a PCR cycler with heated lid to release the tagmented particles into solution.
(xiii) Add 15 µL 0.67% Triton-X and briefly vortex on full speed.
▲ **CRITICAL STEP** SDS will inhibit PCR even after dilution down to 0.01%, but addition of excess non-ionic detergent such as Triton-X100 neutralizes the SDS.

PCR amplification ● Timing 40 min

36 Add 2 µL of 10 µM universal or barcoded i5 primer + 2 µL of 10 µM uniquely barcoded i7 primers, using a different barcode for each sample. Indexed primers designed by Buenrostro et al.[33] are listed in Supplementary Table 1.

37 Move tubes to an aluminium block on ice. Add 25 µL NEBNext HiFi 2× PCR Master mix and perform PCR with the conditions below:

Cycle number	Denature	Anneal	Extend	Final
1			58 °C, 5 min 72 °C, 5 min	
2	98 °C, 45 s			
3–14	98 °C, 15 s	63° C, 10 s		
15			72 °C, 1 min	
16				8 °C, hold

▲ **CRITICAL STEP** To minimize the contribution of large DNA fragments, PCR should be for 12 cycles, with a 10 s 60–63 °C combined annealing/extension step.
▲ **CRITICAL STEP** Tagmentation leaves a gap between each 3′ end of the insert and the reverse mosaic end oligonucleotide. Step 1 of the PCR is required to displace the oligonucleotide while extending the 3′ ends, producing blunt-ended adapter insert fragments. Hot-start polymerases are not active during this critical extension step and should not be used. We combined annealing/extension to minimize the amplification of large fragments.
▲ **CRITICAL STEP** The cycle times are based on using conventional Peltier cycler (e.g., BioRad/MJ PTC-200), and the 3 °C/sec ramp rate of this machine allows sufficient time for annealing as the sample cools from 98 to 60 °C. Use of a rapid cycler with faster ramp rates will require adjustment to ensure extension.

Post-PCR cleanup ● Timing 30 min

38 Remove tubes from the cycler and add 1.3 volume (65 µL) Ampure XP beads, mixing by pipetting up and down ~10×.

39 After a quick spin (~100*g*, 1 s, room temperature), allow the mixtures to sit at room temperature for 5–10 min.

40 Place on a magnet stand and allow the mixtures to clear before carefully withdrawing liquid. While still on the magnet stand add 200 µL 80% ethanol.

41 While still on the magnet stand, withdraw the liquid and add 200 µL 80% ethanol.

42 Withdraw the liquid, and after a quick spin (~100*g*, 1 s, room temperature) remove the remaining liquid with a 20 µL pipette.
▲ CRITICAL STEP Overdrying can reduce recovery of DNA, so continue to the elution step within 5 min.
43 Remove from the magnet stand, add 22 µL 10 mM Tris-HCl pH 8 and vortex on full speed.
44 After 5 min place on magnet stand and allow the mixtures to clear.
45 Remove liquid to a fresh 0.5 mL tube with a pipette.

Library pooling and DNA sequencing ● Timing 1 d

46 Determine the size distribution of libraries by capillary electrophoresis (e.g., Agilent 4200 TapeStation), using 2 µL following the manufacturer's instructions. For mixing single-tube samples with load buffer use a low-volume (e.g., 2 µL of sample) pipettor to avoid injecting bubbles.
? TROUBLESHOOTING
47 (Optional) Quantify the library yield for a dsDNA-specific assay, such as Qubit following the manufacturer's instructions.
▲ CRITICAL STEP Molarity estimates are based on the capillary electrophoresis profile by choosing the region between 175 and 1,000 bp and using the value reported as 'Region Molarity (pmol/L)'.
▲ CRITICAL STEP Due to the very low background with CUT&Tag, typically 3 million mapped paired-end reads per sample suffices, even for the human genome. For maximum economy, we mix up to 96 barcoded samples per lane on a two lane flow cell, and perform paired-end 25 × 25 bp sequencing, which is sufficient for high-confidence mapping with high-affinity histone modification antibodies. Single-end sequencing is not recommended for CUT&Tag, as it sacrifices resolution and fragment size information.
48 Pool libraries with compatible barcodes in equimolar amounts to achieve similar read counts.
(Optional) If necessary, reduce the volume by Speedvac to obtain the molar concentration and volume required by the sequencing facility (e.g., 2 nM)
(Optional) If there is not enough of a very low-yield sample to achieve an equimolar amount in a pool, use the whole sample.
49 Repeat Steps 38–45.
50 Recheck libraries by capillary electrophoresis to make sure that primers have been sufficiently depleted and to obtain a final concentration estimate.
▲ CRITICAL STEP This also provides a length distribution for the pool, which is used to estimate the molar concentration based on a Qubit measurement. The two molar concentration estimates should be similar.
51 Perform paired-end Illumina sequencing on the barcoded libraries using an Illumina HiSeq 2500 or other massively parallel DNA sequencer following the manufacturer's instructions.

Data processing and analysis ● Timing 1 d (variable)

52 Align paired-end reads using Bowtie2 version 2.2.5 (http://bowtie-bio.sourceforge.net/bowtie2/index.shtml) with options: `--end-to-end --very-sensitive --no-unal --no-mixed --no-discordant --phred33 -I 10 -X 700`. For mapping *E. coli* carryover fragments for calibration[27], we also use the `--no-overlap --no-dovetail` options to avoid cross-mapping of the experimental genome to that of the carryover DNA.
? TROUBLESHOOTING
53 A Unix-compatible script for processing CUT&RUN and Tag spike-in and *E. coli* carryover data is available from GitHub (https://github.com/Henikoff/Cut-and-Run). We use the Picard version 2.15 'MarkDuplicates' command (https://broadinstitute.github.io/picard/index.html) to mark presumed PCR duplicates and to estimate library size.
? TROUBLESHOOTING
54 (Optional) Call peaks using SEACR[21], MACS2 (ref. [34]) or other peak-calling program. The SEACR peak-caller was designed for low-read-count, low-background paired-end sequencing data of the type obtained using CUT&RUN and CUT&Tag and is available as a public web server: https://seacr.fredhutch.org or as scripts from https://github.com/FredHutch/SEACR/. Calculate the fraction of reads in peaks (FRiPs)[35] to evaluate signal-to-noise ratio.
? TROUBLESHOOTING

Troubleshooting

Troubleshooting advice can be found in Table 1.

Table 1 | Troubleshooting table

Steps	Problem	Possible reasons	Solutions
20	Beads clump and cannot be disaggregated	Cells lyse	Small clumps (2–5 cells) do not cause problems; but for large clumps reduce the number of cells or treat nuclei more gently
	Beads stick to the walls or cap of the tube and cannot be resuspended	Beads get out of suspension during incubation and dry	Use low-bind tubes. Make sure that beads are immersed in the buffer during incubations
46	No insert for positive control is detected by capillary electrophoresis	May indicate PCR failure	Make sure that sample DNA is free from PCR inhibitors. Make sure that PCR reaction works using a positive control
	Little or no yield of insert	Expected for low cell number samples or rare epitopes, but may indicate antibody failure	Replace antibody. Antibody binding may be tested by IF
	A smear covering a large region, but no nucleosomal ladder is detected by capillary electrophoresis	*E. coli* DNA tagmentation due to high concentration of pA-Tn5 or very low cell number or rare epitope	Decrease pA-Tn5 concentration If possible, start with more material Replace antibody
52	The majority of reads map to *E. coli* genome	*E. coli* reads are expected to increase with decreasing cell numbers or rare epitopes, but may indicate antibody failure	Replace antibody. Antibody binding may be tested by IF
53	>50% Duplicates	Too few cells or rare epitope	Increase cell number
54	Signals are weak and FRiPs are low	High background caused by a weak antibody or unstable binding of target protein	Sequence more deeply

Timing

Day 1, cells to library
Steps 1–4, prepare concanavalin A beads: 15 min
Steps 5–16, prepare nuclei, optionally fix and cryopreserve: 1 h
Steps 17–19, bind nuclei to ConA beads: 15 min
Steps 20 and 21, bind primary antibody: 1 h 15 min
Steps 22–28, bind secondary antibody: 45 min
Steps 29–34, bind pA-Tn5 adapter complex: 1 h 15 min
Step 35A, tagmentation with DNA extraction: 3 h
Step 35B, tagmentation and particle release: 2 h 30 min
Steps 36 and 37, PCR amplification: 40 min
Steps 38–45, post-PCR cleanup: 30 min
Day 2, library pooling and DNA sequencing
Steps 46–51, library preparation and sequencing: 2 d
Day 3 (variable), data processing and analysis
Steps 52–54, ≥1 d

Anticipated results

High CUT&Tag robustness with low DNA sequencing requirements

Improvements in the signal-to-noise ratio can lower the cost of an experiment both by reducing the amount of sequencing needed to identify features and by reducing the size of files that must be transferred, analyzed and stored. For ChIP-seq of histone modifications, such as H3K4me3, H3K36me3 and H3K27me3, 40–50 million is the recommended minimum number of reads for human cells[36]. We previously showed that CUT&Tag for permeabilized cells improves the signal-to-noise ratio and thus reduces sequencing costs relative to ChIP-seq by an order of magnitude[23]. To confirm that our protocol for nuclei also provides an improved signal-to-noise ratio relative to popular chromatin profiling methods, we performed CUT&Tag using both our original protocol for

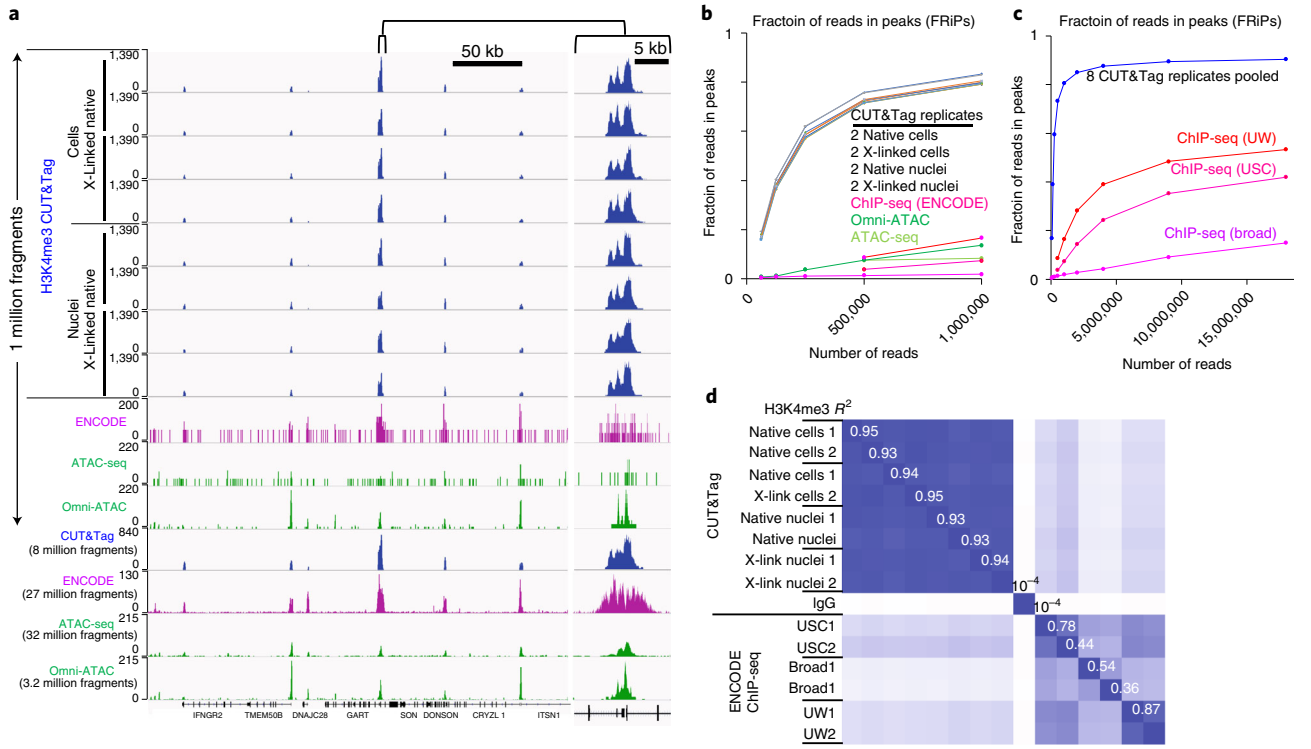


Fig. 2 | CUT&Tag provides high signal-to-noise ratios and reproducibility for native and lightly cross-linked cells and nuclei. H3K4me3 CUT&Tag was performed on native and cross-linked cells and nuclei from the same batch on two different occasions (biological replicates 1 and 2 using the DNA extraction option for nuclei). Barcoded libraries were mixed with 70 other barcoded libraries and sequenced, yielding at least 1 million fragments. **a**, H3K4me3 CUT&Tag (blue), ENCODE H3K4me3 ChIP-seq (magenta GSM733680), ATAC-seq (GSM2695560) and Omni-ATAC (SRX2894091-2) tracks (green) for K562 cells. **b**, MACS2 was used to call peaks for datasets shown in **a** and additional datasets from ENCODE (H3K4me3) and GEO (Omni-ATAC) to calculate the fraction of reads in peaks (FRiPs). The multicolored curves without marker dots represent the 8 CUT&Tag replicates. **c**, The 8 CUT&Tag datasets were pooled for FRiPs comparisons to ChIP-seq for up to 18 million fragments. **d**, A correlation matrix of MACS2 narrow peak calls for biological replicates representing the 8 CUT&Tag datasets and pooled IgG datasets and the six ENCODE datasets from three laboratories. Peaks were called using MACS2 on the pooled CUT&Tag H3K4me3 resulting in 12,224 peaks, from which 223 were removed because of high IgG density. Pairwise correlations of mean normalized counts were calculated over a span of ±150 bp around each summit. Numbers along the diagonal are R^2 values between successive rows; for example between IgG and USC1 $R^2 = 10^{-4}$, between USC1 and USC2 $R^2 = 0.74$, and between USC2 and Broad1 $R^2 = 0.44$.

cells[23] and our current protocol for nuclei with an antibody to histone H3K4me3, a mark of active promoters. Human K562 cells were washed in PBS and half of the culture was used to prepare nuclei. The cells and nuclei were each split such that half of each suspension was lightly fixed (0.1% formaldehyde for 2 min at room temperature)[37]. H3K4me3 CUT&Tag was performed on cells permeabilized by digitonin followed by extraction using our previous protocol[23] (option A), then barcoded during PCR amplification. The same experiment was also done using 0.2% formaldehyde. All eight samples were pooled with 70 other barcoded samples and subjected to paired-end 25 × 25 sequencing on a single flow cell. Examination of a representative 300-kb region shows clean promoter peaks essentially identical for all eight samples when only 1 million fragments were mapped (Fig. 2a). This demonstrates that CUT&Tag provides highly reproducible data for native or lightly fixed cells or nuclei.

For comparison, we sampled 1 million fragments from 6 ENCODE H3K4me3 ChIP-seq datasets from three different laboratories and ATAC-seq and Omni-ATAC datasets obtained from GEO, which showed weak barely detectable peaks over a sparse background for ChIP-seq and ATAC-seq. When ~30 million ChIP-seq and ATAC-seq fragments were mapped, the same promoter peaks that were seen in each of the 1 million fragment CUT&Tag tracks were confirmed by the other methods. To extend this comparison genome-wide, we called peaks using MACS2 and calculated the FRiPs, a measure of the signal-to-noise ratio adopted by the ENCODE project[35]. Based on FRiPs, CUT&Tag shows nearly identical performance for all 8 samples (two biological replicates from native and lightly cross-linked cells and nuclei), confirming the high robustness of CUT&Tag regardless of which protocol is used (Fig. 2b). In contrast, ChIP-seq and Omni-ATAC datasets were much less suitable

for peak-calling when downsampled to 1 million reads, with both ChIP-seq and Omni-ATAC showing an order-of-magnitude lower FRiPs relative to CUT&Tag. We also pooled the fragments from the 8 CUT&Tag datasets and downsampled from the pool to compare FRiP curves at high read depths, and observed a FRiP of 0.90 for CUT&Tag versus 0.15–0.53 For ChIP-seq and Omni-ATAC (Fig. 2c). Reproducibility of H3K4me3 CUT&Tag biological replicates was also much better than that for ChIP-seq, with R^2 values for narrow peaks called by MACS2 ranging from 0.93– 0.95 compared to 0.39–0.87 for H3K4me3 ChIP-seq peaks from three ENCODE laboratories (Fig. 2d).

CUT&Tag has also been implemented for single-cell chromatin profiling, and we previously reported a dataset consisting of 807 H3K27me3 single-cell profiles, which we find outperforms a recent single-cell ChIP-seq method[8] (Box 1).

Box 1 | scCUT&Tag compared to single-cell ChIP-seq

We previously showed that CUT&Tag is suitable for high-throughput single-cell profiling using the Takara ICELL8 nanowell platform[23]. Meanwhile, another group introduced a high-throughput single-cell version of native ChIP-seq (scChIP-seq) using a microfluidic device[8]. That study included scChIP-seq experiments on Jurkat and Ramos haematopoietic cancer cell lines using an H3K27me3 rabbit monoclonal antibody, the same antibody as we had used for scCUT&Tag of K562 cells and also a cancer cell line of haematopoietic origin. Direct comparison of a representative H3K27me3 domain shows that the signal-to-noise ratio is similar between bulk and scCUT&Tag, but is ~10-fold lower using scChIP-seq for both Jurkat and Ramos cells (Fig. 3a). The efficiency of scCUT&Tag was also better than that for scChIP-seq, with a ~6-fold higher median fragment-per-cell ratio over the distribution of cells reported in both studies. When we used MACS2 to call broad peaks on these domains genome-wide, we found close correspondence between bulk and scCUT&Tag, with FRiPs leveling off at 1 million fragments, which is ~30-fold higher than that for scChIP-seq at 1 million fragments (Fig. 3b). The median level of reads per cell was ~6-fold higher for scCUT&Tag than for scChIP-seq (Fig. 3c). We conclude that scCUT&Tag on the ICELL8 is very suitable for efficient single-cell profiling, and anticipate that CUT&Tag will be soon adopted for other high-throughput single-cell platforms.

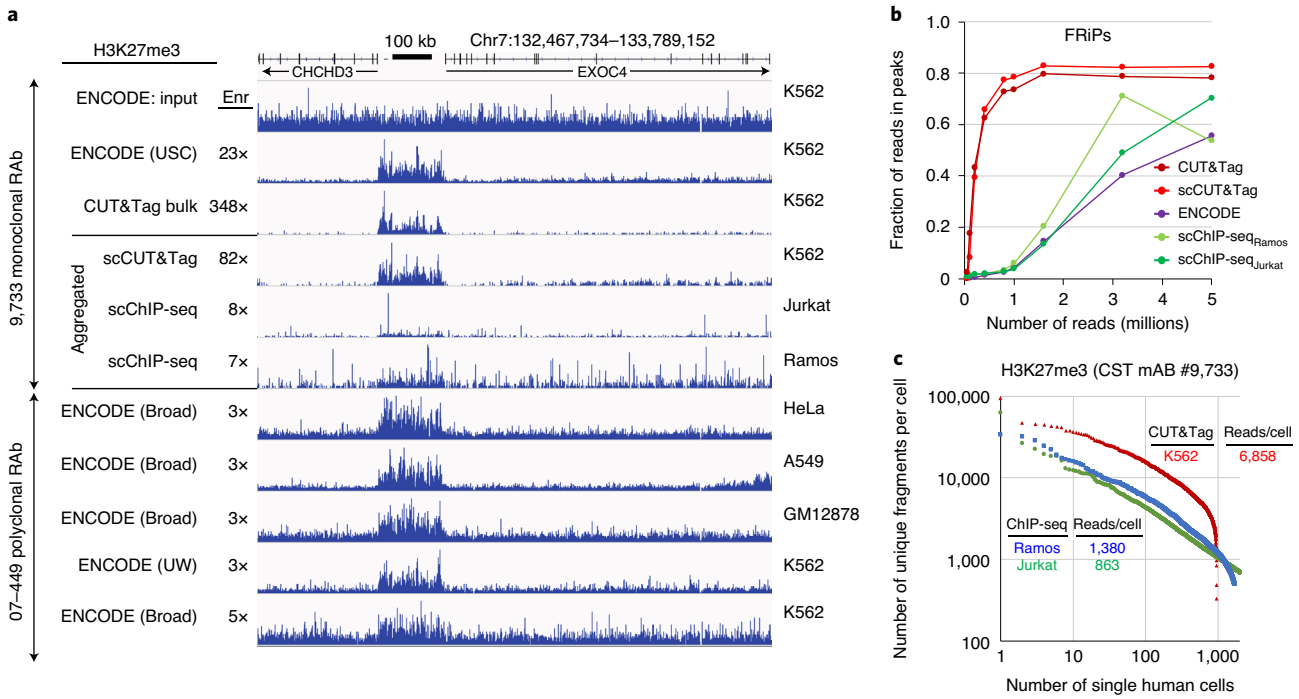


Fig. 3 | Comparison of scCUT&Tag to single-cell ChIP-seq. The same monoclonal antibody was used for H3K27me3 scCUT&Tag and scChIP-seq allowing a direct comparison of aggregated single-cell datasets. **a**, Tracks from a representative region of the human genome. Although different haematopoietic cancer cell lines were used, this region shows similar profiles for diverse cancer and normal lines (indicated on the right). To quantify approximate signal-to-noise ratio differences, we calculated the fold enrichment over the H3K27me3-enriched domain (132,768,919–132,935,822) relative to that for the flanking regions (132,467,734–132,768,918 and 132,935,823–133,789,152) for each track relative to the fold enrichment for the ENCODE USC K562 input track ('Enr' numbers for each track). **b**, Noisiness of scChIP-seq data relative to CUT&Tag data is confirmed by FRiP analysis. **c**, Knee plot showing the read/cell distribution estimated from aggregate data using the Picard Mark Duplicates program. Medians are indicated, where for scCUT&Tag all cells were scored and for scChIP-seq cells below a threshold were not scored.

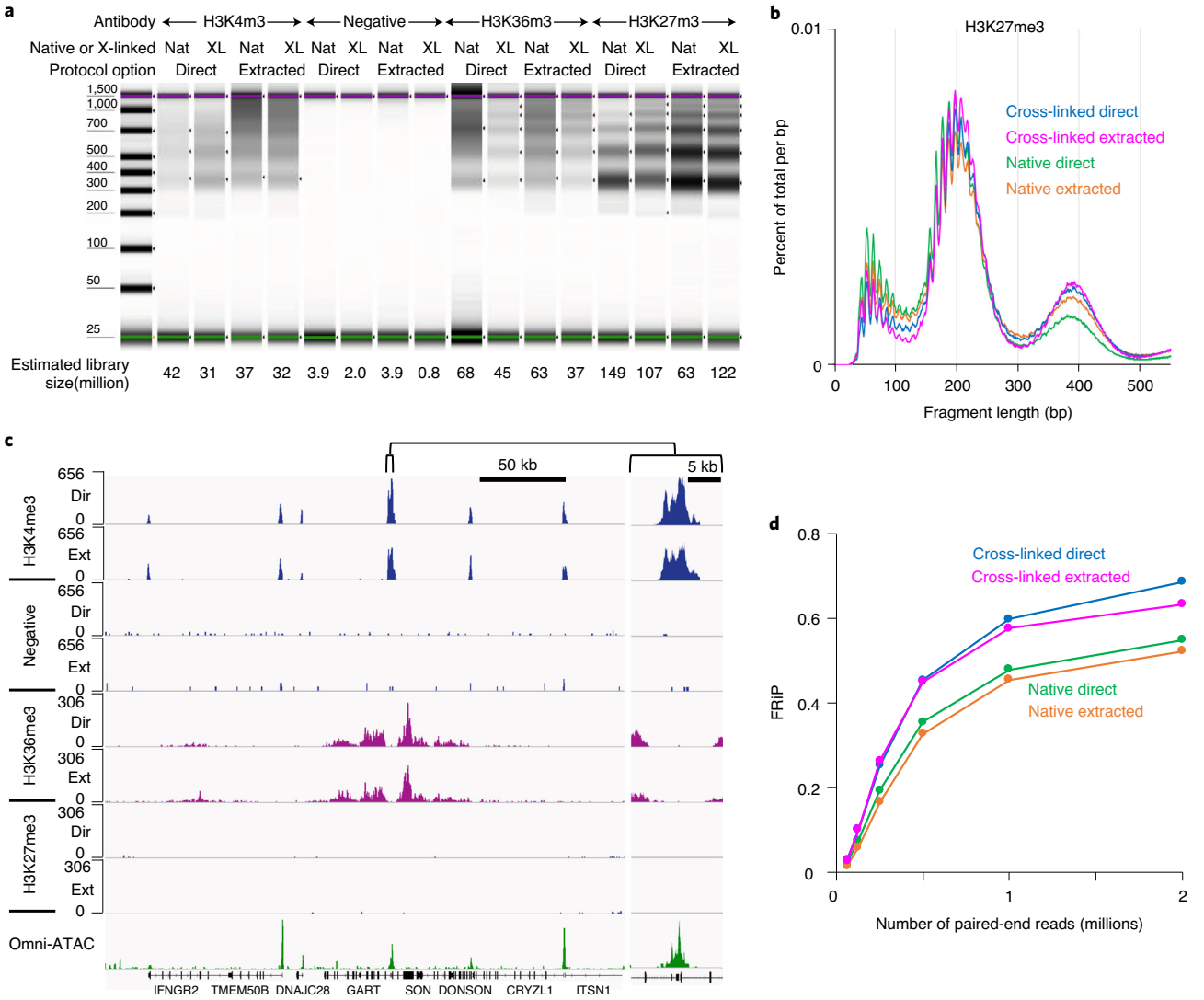


Fig. 4 | Similar results are obtained using DNA extraction and single-tube CUT&Tag options. a, Image of a capillary electrophoretic gel for a typical experiment. Native and cross-linked nuclei were prepared and frozen in advance. Samples were thawed, mixed with beads and ~120,000 nuclei were aliquoted into each PCR tube. All steps through TapeStation analysis were performed in 1 d in parallel for both the extraction and single-tube (direct) options. The 16 samples were mixed in equimolar amounts with 56 other samples and sequenced (paired-end 25 × 25), yielding a median of 2.7 million mapped reads per sample. Estimated library size is indicated below. **b**, Length distribution for sequenced fragments from H3K27me3 CUT&Tag. The striking 10-bp sawtooth pattern that diminishes with length suggests a tagmentation preference for one surface of the DNA double-helix at a fixed distance on and around the bound particle. **c**, The same 300-kb GART-SON region displayed in Fig. 2 is shown for the direct-to-PCR single-tube samples. The negative control had been incubated with a validated H3K27ac mouse monoclonal antibody followed by the anti-rabbit secondary antibody, which suppressed the signal. **d**, Mapped fragments were sampled and narrow peaks were called using MACS2 with a *p* value of 10^{-5} and FRiP values were calculated.

From nuclei to sequencing-ready library in a single tube

Our original protocol uses organic extraction to prepare tagmented samples for PCR. We have developed a protocol that proceeds from tagmentation directly to PCR, releasing pA-Tn5 from the DNA using a low concentration of SDS in a small volume and incubating for 1 h at 58 °C, which also reverses the cross-links (option B). Triton-X100 is added to the beads to neutralize the SDS, which would otherwise inhibit Taq polymerase. This is followed by addition of PCR primers and master mix. Post-PCR cleanup is performed by addition of SPRI beads, such that the sequencing-ready library is withdrawn from the same tube used for all previous steps. Results using this method are comparable to results using organic DNA extraction (Fig. 4).

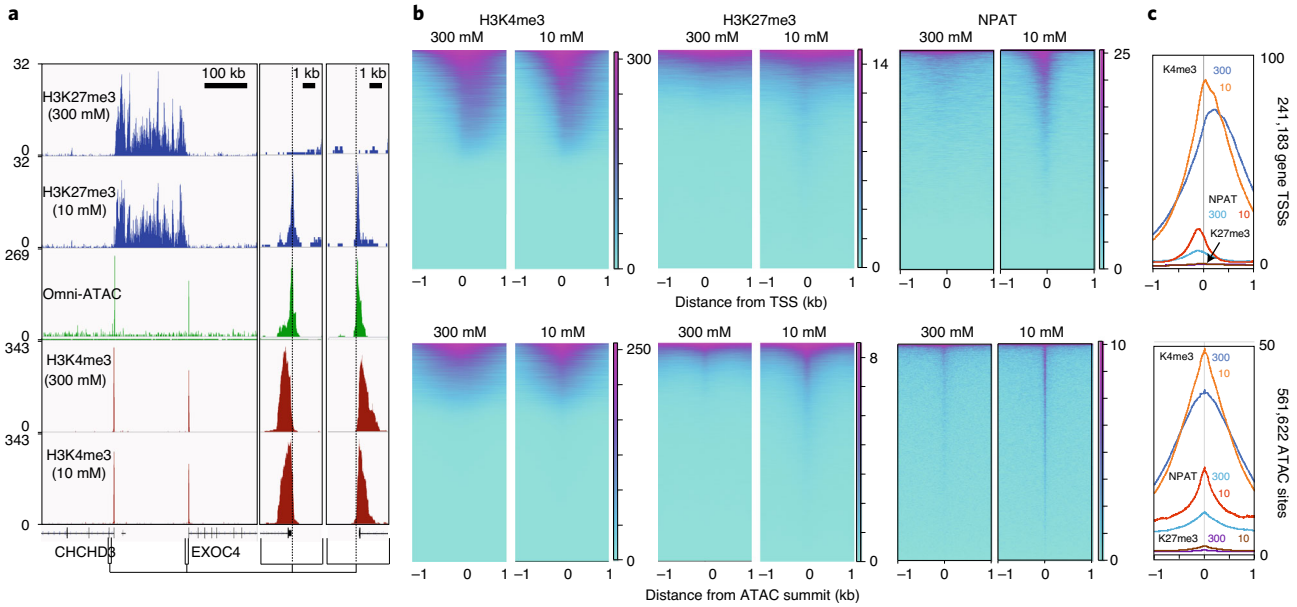


Fig. 5 | Suppression of accessible DNA tagmentation. a, A representative region spanning a large H3K27me3 domain flanked by oppositely oriented promoters marked by H3K4me3 nucleosomes just downstream of the TSSs in K562 cells. CUT&Tag with DNA extraction was performed on ~70,000 lightly cross-linked frozen and thawed nuclei per sample using the DNA extraction option, except that the third wash and tagmentation steps were done in 10 mM TAPS at pH 8.5. This resulted in peaks over the TSSs for H3K27me3 that are nearly absent in the presence of 300 mM NaCl. For clarity, 5 kb regions around both promoters are expanded on the right, revealing that the low-salt peaks align with the Omni-ATAC peaks and the promoters, but are offset from the H3K4me3 nucleosomes downstream (dashed lines). **b**, Heatmap representations and **c**, average plots of 24,183 annotated promoters (top) and 56,622 ATAC-seq MACS2 peak summits (bottom) for CUT&Tag histone modifications and the NPAT chromatin protein, showing genome-wide suppression of accessible DNA peaks when tagmentation is performed in the presence of 300 mM NaCl. Heatmaps were separately ordered by signal over the displayed region using Deeptools.

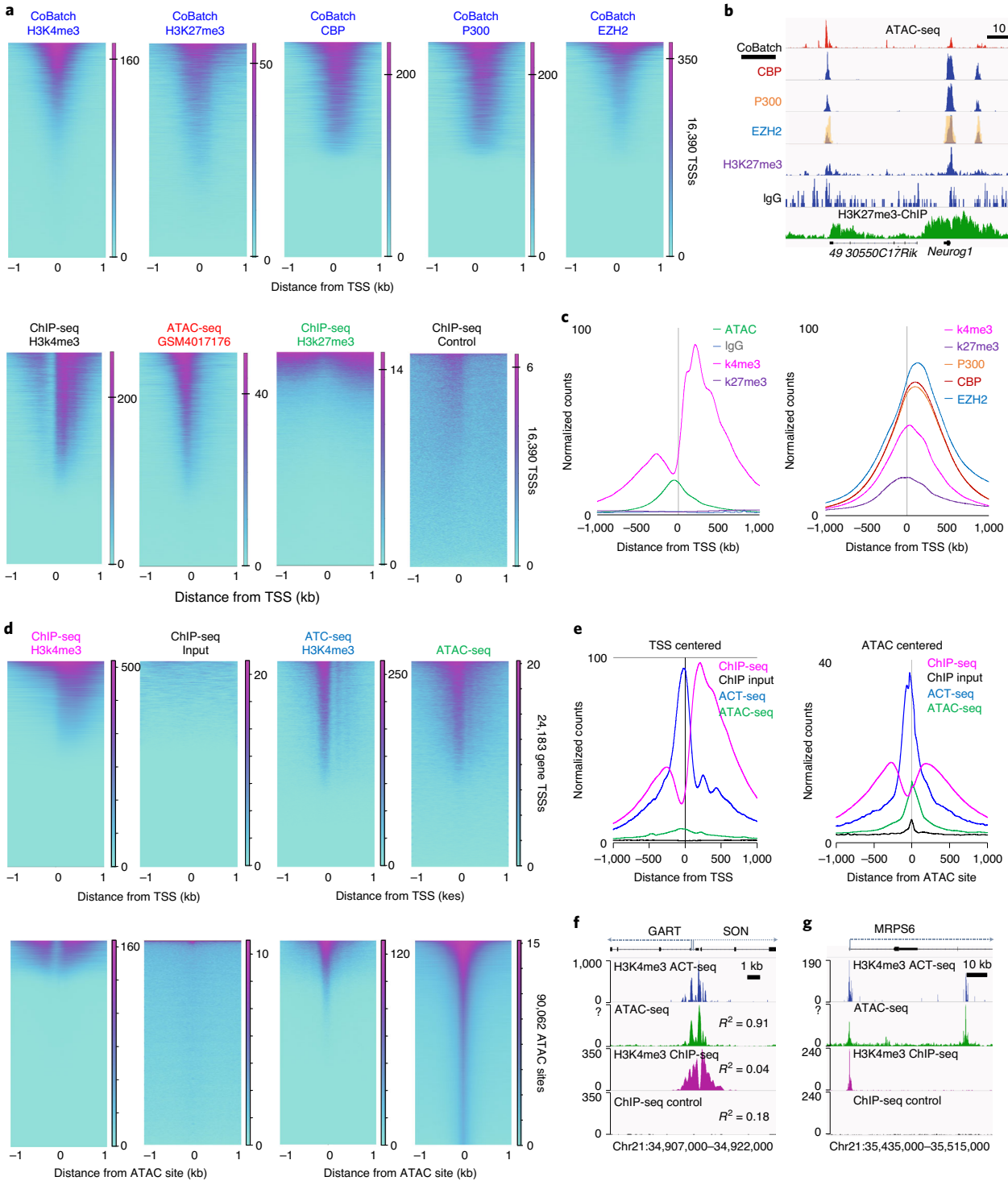
Box 2 | Accessible DNA detection by CoBATCH and ACT-seq

Analysis of published data from two groups demonstrates the possibility of accessibility artifacts in Tn5-based profiling, previously described by Harada et al.[24]. One recent publication applied CUT&Tag in a single-cell-compatible format called CoBATCH[26]. This study mapped multiple activating and repressing chromatin proteins and histone modifications, but with strikingly similar results for all proteins (Fig. 6a,b). CoBATCH mapping of H3K27me3 is particularly telling, as this is disagrees with mapping of the same modification by ChIP-seq. Instead, each peak in the CoBATCH tracks corresponds to an ATAC-seq profiling peak regardless of which antibody was used. This is most likely due to the low salt concentrations this study used before and during tagmentation, as the ratio between antibody-tethered and accessibility tagmentation decreases at reduced salt concentration (Fig. 5). It is also apparent that CoBATCH conditions improve the detection of accessible DNA in mouse embryonic stem cells (mESCs; Fig. 6c), as these sites are better represented with much lower backgrounds than ten ATAC-seq profiles for mESCs from different laboratories with 5–25× more reads (Supplementary Fig. 1).

ACT-seq is a strategy similar to CUT&Tag, except that the antibody is mixed together with pA-Tn5 and applied to permeabilized cells with a single in situ binding reaction in a buffer containing 150 mM NaCl[25]. Analysis of the ACT-seq dataset from GEO for aggregate H3K4me3 single-cell data from human HEK293 cells shows that the ACT-seq signal differs from the H3K4me3 ENCODE ChIP-seq signal for HEK293 cells in that it is not centered over the H3K4me3 nucleosome, but rather in the adjacent accessible DNA site (Fig. 6d). As for CoBATCH, the signal-to-noise ratio of the accessible DNA signal is much better than that of ATAC-seq for the same HEK293 cell type (Fig. 6e). When mapped with base-pair resolution, the offset of the ACT-seq signal correlates strongly with the ATAC-seq signal but shows essentially no similarity with the H3K4me3 ChIP-seq signal (Fig. 6f). ACT-seq peaks are seen not only at promoters marked by H3K4me3 using ChIP-seq, but also at non-promoter sites that lack a ChIP-seq signal (Fig. 6g).

CUT&Tag stringent salt conditions suppress accessible DNA detection

CoBATCH and ACT-seq are alternative versions of the CUT&Tag strategy, but there are key differences that have implications for suitability in different applications. We have found that stringent binding conditions during pA-Tn5 tethering and in subsequent washes and during tagmentation are critical to limit the affinity of Tn5 for exposed DNA. Such affinity, if not properly controlled, leads to tagmentation of exposed DNA in an ATAC-like profile that can be mis-interpreted as a genomic profile of a chromatin epitope. We find that performing all steps from pA-Tn5 binding through tagmentation in the presence of 300 mM NaCl simply and effectively suppresses this artefact (Fig. 5a). Heatmaps aligned to TSSs and ATAC-seq sites show close correspondence for H3K4me3, a positive



control that marks nucleosomes just downstream of active promoters when tagmentation is done using either 300 or 10 mM monovalent ionic conditions (Fig. 5b). However, tracks and heatmaps for H3K27me3, an abundant histone modification that marks transcriptionally silent domains, show a weak signal directly over regulatory sites for 10 mM monovalent ionic conditions. For NPAT, a transcription factor that marks <100 annotated sites mostly within the histone gene clusters on human chromosomes 1 and 6, the artefact is very conspicuous when tagmentation is performed in 10 mM monovalent ionic conditions, and is strongly suppressed by 300 mM NaCl (Fig. 5c). Taken

◀ **Fig. 6 | CoBATCH and ACT-seq peaks correspond to ATAC-seq peak summits genome-wide. a**, Heatmaps are similar between ATAC-seq summits and CoBATCH H3K27me3 signals but correspond to depleted H3K27me3 ChIP-seq signals in mESCs. Likewise, CoBATCH CBP, P300 and EZH2 summits correspond to one another genome-wide with an ~10-fold larger dynamic range than is seen for ATAC-seq (0 to 200–350 for CoBATCH, 0–20 for ATAC-seq). For each dataset, heatmaps are ordered by decreasing normalized count density. The ATAC-seq track shown is better than average based on comparing mESC ATAC-seq tracks from eight different laboratories (Supplementary Fig. 1). **b**, Representative examples from tracks shown in Fig. 2c of ref. [19]. The EZH2 track from the figure image (yellow) is superimposed over the track of the same region reproduced from the data in GEO, confirming correspondence between the published CoBATCH image and the source data used here. Profiles for CBP, P300 and EZH2 closely correspond to one another and to ATAC-seq peaks, although with much lower background, and lack the broad domains seen for H3K27me3 ChIP-seq profiles. **c**, Average plots of the data shown in **a**. **d**, Comparison of H3K4me3 ChIP-seq to ACT-seq and ATAC-seq heatmaps. ACT-seq shows a chromatin accessibility profile with a ~10-fold larger dynamic range than is seen for ATAC-seq (0–250 for ACT-seq, 0–20 for ATAC-seq). **e**, Average plot of the dataset shown in the top panels of **d**. **f**, A representative region showing bidirectional housekeeping promoters and R^2 Pearson correlation coefficients over the region between ACT-seq, ATAC-seq and ChIP-seq from human K562 cells. **g**, A nearby representative gene region showing the promoter marked by ACT-seq, ATAC-seq and H3K4me3 ChIP and an intronic region marked by ACT-seq and ATAC-seq, but not by ChIP-seq. Heatmaps were separately ordered by signal over the displayed region using Deeptools.

together, these results imply that rare epitopes bound at regulatory elements are most susceptible to the artefact, which is suppressed by 300 mM NaCl. However, this high salt concentration may also reduce the signal of loosely-bound factors by displacing them from chromatin.

Suppression of tagmentation of accessible DNA is essential for accurate antibody-dependent CUT&Tag chromatin profiling. We find that variations in the basic CUT&Tag strategy, such as that used in CoBATCH and ACT-seq (Box 2) detect accessible DNA with high efficiency. The improved signal at accessible sites for ACT-seq and CoBATCH methods relative to ATAC-seq from many laboratories is not fully understood, but may be due to the wash steps following pA-Tn5 tethering that limit background tagmentation of the genome.

In conclusion, the ability to obtain high-quality chromatin profiles easily and at low cost by using our CUT&Tag protocol enables studies that might otherwise be cost prohibitive. For example, chromatin profiling of small numbers of selected cells provides an attractive alternative to single-cell profiling for developmental studies, where applying CUT&Tag to cells purified by fluorescence-activated cell-sorting[38], laser-assisted microdissection[39], INTACT[40] and other cell-isolation methods promises to provide highly robust cell function information that can be used to characterize cell types directly.

Reporting Summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Publicly available datasets analyzed in this work are available in Supplementary Note 1. All sequencing data generated in this study have been deposited in GEO under accession GSE145187.

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Author contributions

H.S.K.-O. and S.H. developed the protocol with input from K.A and D.H.J. S.H. performed the experiments, and with J.G.H. analyzed the data. S.H. and K.A. wrote the manuscript with input from H.S.K.-O, D.H.J., and J.G.H.

Competing interests

H.S.K.-O. and S.H. have filed patent applications related to this work.

Additional information

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Methods

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☒	☐ Flow cytometry
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Antibodies

Antibodies used	NPAT: Thermo Invitrogen cat# PA5-66839 lot# SL2495022A H3K27me3: Cell Signaling Technology cat# 9733 H3K4me3: Active Motif cat# 39159 lot# 22118006 H3K27ac: Thermo cat# MA5-23516 (mouse) primary H3K36me3: Epicypher cat# 24213-0031 lot# 18303001 IgG: Guinea pig anti-rabbit Antibodies-Online ABIN101961 lot# 200-12190001
Validation	Commercial antibodies were validated by the companies.

Eukaryotic cell lines

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Cell line source(s)	K562: ATCC, catalog# CCL-243. H1: WiCell WA01-WB35186.
Authentication	none were authenticated
Mycoplasma contamination	Negative for mycoplasma
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Genome browser session (e.g. UCSC)	n/a

Methodology

Replicates	At least 2 replicates were performed. Methodological protocol is the topic of this manuscript.
Sequencing depth	All Experiments were paired-end. Sequencing depths and sampling is reported in the manuscript.
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